

LOC148158 + ATP/AMP

PDB:3C5E

Revision

Revision Type:created

Revised by:created

Revision Date:created

Entry Clone Accession:NM_182617 Variant

Entry Clone Source:Origene

SGC Clone Accession:

Tag:N-terminal, TEV cleavable hexahistidine tag.Tag sequence: mhhhhhssgvdlgtenlyfq(*)sm

Host:*Trichoplusia Ni* (High five)

Construct

Prelude:

Sequence:

mghhhhhhssgvdlgtenlyfqsmSLQWGHQEVPAKFNFASDVLHDWADMEKAGKRPPSPALWWVNGKGKELMWNFRELSSENSQQAANVLSGACGLQRGDRVAVVLPRVPEWWLVILGCIRAGLIFMPGTIQMKSTDILYRLQMSKAKAIVAGDEVIQEVDTVASECPSLRIKL
LVSEKSCDGLNFKKLLNEASTTHHCVETGSQEASAIYFTSGTSGLPKMAEHSYSSLGLKAKMDAGWTGLQASDIMWTISDTGWILN
ILCSLMEPWALGACTFVHLLPKFDPLVILKTLSSYPIKSMMGAPIVYRMLLQQDLSSYKFPHLQNCVTVGESLLPETLENWRAQTGL
DIRESYQTETGLTCMVSKTMKIKPGYMGTAASCYDVQIIDDKGNVLPPTGEGDIGIRVKPIRPIGIFSGYVDNPDKTAANIRGDFW
LLGDRGIKDEDGYFQFMGRADDIINSSGYRIGPSEVENALMEHPAVVETAVISSPDPVRGEVVKAFVVLASQFLSHDPEQLTKELQQ
HVKSVTAPYKYPRKIEFVLNLPKTVTGKIQRAKLDRDKEWKMSGKARAQ

Vector:pFBOH-LIC-Bse

Growth

Medium:

Antibiotics:

Procedure: High five cells were grown in Sf900II medium supplemented with 1% FCS at 27°C. Cells were infected at a density of 2x10⁶/ml with recombinant baculovirus (virus stock P2; 1ml of virus stock/1l of cell culture). 72 hours post-infection the cultures were collected and centrifuged for 30min at 2000rpm. The cell pellet was resuspended in cold PBS and centrifugation was repeated. The pellet was resuspended in lysis buffer (50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol + EDTA-free Complete (1 tablet/50ml)) and frozen at -80°C until purification.

Purification

Procedure

Purification: Step 1: Ni-affinity, Ni-Sepharose - (GE Healthcare) purification in batch Step 2: Superdex 200 Column, HiPrep 16/60 (Amersham) Step 3: Ion exchange - 5ml HiTrap Q Sepharose

Buffers: Start buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP; Washing buffer: 50mM HEPES pH 7.5, 500mM NaCl, 10mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP; Elution buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5% glycerol, 250mM Imidazole, 0.5mM TCEP; GF buffer: 10mM HEPES pH 7.5, 500mM NaCl, 5% glycerol, 0.5mM TCEP; IEX buffers: Buffer A: 50mM Tris pH 9.0; Buffer B: 50mM Tris pH 9.0, 1M NaCl

Procedure: The cell extract was incubated with Ni-Sepharose (0.250 µl of resin/lysate from 1l of culture) during 1h at 4°C with gentle rotation. The resin was centrifuged at 1000g for 5min at 4°C, and washed 4x with 50ml of washing buffer. Resin was loaded on the gravity column and protein was eluted in 4 elution fractions (5ml each). Protein fractions were analysed by SDS-PAGE. Target protein containing fractions were concentrated using Amicon Ultra-15 concentrators with 10kDa cut-off, and purified on a gel filtration column (Superdex 200) on an Akta Purifier System. LOC148158 containing fractions were diluted with IEX buffer A to a concentration of NaCl of 50 mM. The protein was loaded onto a HiTrap Q Sepharose column, and eluted with a NaCl gradient (50-300mM). Fractions containing protein were analysed by SDS-PAGE.

Concentration and buffer exchange: Using Amicon Ultra-15 concentrators with 10kDa cutoff, the sample was concentrated to 20mg/ml. Concentrations were determined from the absorbance at 280 nm using a NanoDrop spectrophotometer.

Extraction

Procedure

Lysis buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5mM Imidazole, 5% glycerol + EDTA-free Complete (1 tablet/50ml).

The thawed cellular lysates were supplemented with Igepal CA 630 (Fluka) at a final concentration of 0.05%, and benzonase (Novagen) (25u/10ml of lysate). Cells were broken using a Dounce homogeniser, followed by centrifugation for 45 min at 20.000rpm at 4°C.

Concentration: Using Amicon Ultra-15 concentrators with 10kDa cutoff, the sample was concentrated to 20mg/ml. Concentrations were determined from the absorbance at 280 nm using a NanoDrop spectrophotometer.

Ligand

ATP/AMPMassSpec: The calculated mass of the construct was 63260Da, and the observed mass (ESI-MS) was 63173Da, compatible with an N-terminal methionine deletion and subsequent acetylation at the N-terminus of the protein.

Crystallization: Crystals were grown by vapor diffusion at 20°C in 150nl sitting drops. MgCl₂ was added to a final concentration of 10 mM to the buffer in the well prior to crystallisation. ATP was added to final concentration of 5mM to the protein solution. The drops were prepared by mixing 100nl of protein solution and 50nl of precipitant consisting of 17% PEG 10K; 0.10M (NH₄) acetate; 0.1M BIS-TRIS pH 5.5. Crystals were flash-cooled in liquid nitrogen with 25 % glycerol as cryoprotectant.

NMR Spectroscopy:

Data Collection: Resolution: 1.9Å; X-ray source: Swiss Light source (SLS), beamline X-10.

Data Processing: